Cortical Axon Trajectories and Growth Cone Morphologies in Fetuses of Acallosal Mouse Strains

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Ozaki, H.S., and Wahlsten, D. Cortical axon trajectories and growth cone morphologies in fetuses of acallosal mouse strains. Journal of Comparative Neurology, 1993, 336, 595-604.

Abstract:
Hereditary absence of the corpus callosum (CC) provides an ideal experiment of nature for exploring mechanisms of axon guidance. In this study the prenatal development of CC axons in the acallosal mouse strains BALB/cWah 1 and 129/ ReJ or J was compared with normal hybrid mice by using the lipophilic dyes DiI and DiA. A few I /LnJ mice were also examined. The time of emergence and growth rate of CC axons from four cortical regions (frontal, parietal, temporal, occipital) were normal in acallosal strains. Their CC axons arrived at midplane on schedule but then often looped back to form the longitudinal Probst bundle. The frequency of formation of the Probst bundle was highest for axons from frontal cortex, which arrived at midplane first, and lowest for occipital axons, which arrived last. Once a few CC axons found a path to the other side via the hippocampal commissure, those that arrived later then crossed relatively normally. Some axons from the Probst bundle also managed to traverse midline in this manner. When no CC axons crossed, almost all of them entered the Probst bundle and eventually left it within a few hours to proceed in the ipsilateral white matter, never turning back toward midplane. Growth cones approaching midplane ipsilaterally and those that had crossed midline and entered contralateral white matter, as well as CC axons in the Probst bundle, expressed a normal range of size and complexity. These results demonstrate that the problem with callosal agenesis resides not in the cells of origin or the axons or growth cones themselves but in the substrates of axon guidance at the midsagittal plane.
Key words: axon guidance, inbred strain, corpus callosum, hippocampal commissure, tract tracing

Article:
Through extensive studies performed during the last decade, much progress has been made in understanding the developmental events producing hereditary agenesis of the corpus callosum (CC) seen in several mouse strains (for review, see Wahlsten, '89; Wahlsten and Ozaki, '93). Axons of the mouse CC first cross between the cerebral hemi-spheres in the fetus. Histological comparison with normal fetuses reveals that the principal anomaly of CC formation in acallosal fetuses appears to occur at or near the telencephalic midline; acallosal fetuses suffer from both the presence of a deep cleft in the longitudinal cerebral fissure and a malformation or absence of a transitory layer of subventricular cells extending from the lateral ventricle toward midline, which serves as a developmental matrix for early callosal axons in normal fetuses (Silver et al., '82; Wahlsten, '87). Furthermore, surgical interference with cells near the telencephalic midline prior to arrival of callosal axons produces a brain defect similar to hereditary agenesis of the corpus callosum (Silver et al., '82; Silver and Ogawa, '83; Hankin and Silver, '86), as does prenatal irradiation (Schmidt and Lent, '87; Schneider and Silver, '90). On the basis of these findings, it is currently hypothesized that the anatomical problem with callosal agenesis resides in the substrate for axon growth near midline and that processes of axon guidance in acallosal mice are relatively normal until the fibers approach the midsagittal plane and find that the proper substrates for crossing midplane are missing. It remains to be confirmed, however, that in fetuses of acallosal strain mice callosal axons do actually emerge from their cortical cells of origin and reach midplane on the same schedule as in fetuses of normal strain mice. This is mainly because until recently there have been no convenient and appropriate tract tracing methods for studying axon growth quantitatively in developing fetal brain.

The recent introduction of postmortem fluorescent tracers has greatly improved the observation of axon extension and growth cone morphology in fetuses (Godement et al., '87; Honig and Hume, '89), which allows much stronger statements about normal or pathological development of fetal callosal axons. In a previous study (Ozaki and Wahlsten, '92), through statistical treatments of results obtained with carbocyanine dye labeling techniques, we have established a quantitative standard of normal callosal development in mouse fetuses, which is necessary to judge when and where the first deviation from the normal course of ontogeny occurs in acallosal fetuses. The present study documents prenatal development of cortical axons in acallosal mouse strains, with special emphasis on the timing of the first emergence and arrival at midplane of callosal axons, their behaviors after the deviation from normal ontogeny, and growth cone morphologies along the callosal pathways.

MATERIALS AND METHODS

Mice, mating, and fetuses

Fetuses from the inbred strains BALB/cWah 1 and 129/ ReJ or J were the main focus of this study. Complete absence of the corpus callosum occurs in about 20% to 30% of adults of these strains, but almost all fetuses exhibit severe defects of the telencephalic midline (Wahlsten, '87; Wahlsten and Bulman-Fleming, '90). BALB/cWah 1 mice were bred and maintained at the University of Alberta, whereas 129 mice were obtained from the Jackson Laboratory (Bar Harbor, ME). No differences between the 129 substrains ReJ and J were observed in any phase of this study. One litter of the I /LnJ strain from the Jackson Lab was also examined, but the sample size was obviously too small for statistical evaluation. This interesting strain, which never has even a small fragment of CC present in the adult (Gruber, et al., '91; Livy and Wahlsten, '91), is currently in extremely short supply (we have waited over a year for another shipment) and has very low fertility.

Methods of breeding were the same as those employed by Ozaki and Wahlsten ('92). Briefly, females were checked for presence of a vaginal plug every 4 hours or after being mated overnight, and conception (0.0 day) was defined as the time midway between detection of a plug and the previous plug check. At embryonic ages between E16.4 and E18.1, pregnant mice were deeply anesthetized with sodium pentobarbital (120 mg/kg, intraperitoneal injection), and fetuses were retrieved from the uteri and placed in ice-cold 0.9% physiological saline. After being blotted and weighed, each fetus was perfused through the left ventricle with 3-5 ml of 10 mM phosphate-buffered physiological saline followed by 10-15 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.6).

TABLE 1. Frequencies of Fetuses for Each Strain With Various Numbers of Injection Sites

Injection sites	BALB/cWah1	129/ReJ or J	I/LnJ	Total
4	13	12	0	25
3	20	36	0	56
2	8	48	5	61
1	0	2	0	2
Total	41	98	5	144

Dye application and viewing

After 3 to 5 days in fresh fixative, the cerebral cortex of both hemispheres was exposed and, with the aid of a dissecting microscope and a graticule scale in a 10 × eyepiece, tiny crystals of the dyes (about 50 μm in size; Molecular Probes, Inc.) 1,1' -dioctadecy1-3,3,3' ,3'-tetramethyl-indocar-bocyanine perchlorate (DiI) and 4-(4-dihexadecylamino-styry1)-N-methylpyridinium iodide (DiA) were inserted into the cerebral cortex with the tip of a fine pin. Various combinations of the tracers DiI and DiA were placed in four cortical regions (frontal, parietal, temporal, and occipital cortex), each cerebral hemisphere receiving at most one DiI crystal and one DiA crystal. Table 1 shows the number of fetal brains with various numbers of dye injection sites by strain. Generally, younger fetuses were given more dye injection sites than older ones because axons from different regions were still widely separated. Locations of injection sites were determined by dividing the cerebral cortex into thirds longitudinally, and frontal, parietal, and occipital injection sites were approximately at the center of the anterior, middle, and posterior thirds, respectively. For these three injection sites, caudal distances from the frontal pole were 0.4 to 0.8 mm, 1.2 to 2.0 mm, and 2.4 to 3.2 mm, and lateral distances from the cerebral midline varied from 0.8 to 1.6 mm, 1.0 to 1.8 mm, and 0.8 to 1.6 mm, respectively, depending on size of the brain. The temporal site was in the middle third, 1.4 to 2.4 mm from the frontal pole and 2.0 to 3.0 mm from the midline. After the dye application, the heads were returned to fresh fixative, placed in a 37°C oven for the first

week, and then stored at room temperature for a subsequent 3 to 8 weeks (longer times for older fetuses) in the dark. Incubation of specimens at 37°C was employed to enhance the diffusion of dyes (Senft, '90). These parameters yielded excellent images of callosal axons and growth cones. The brains were dissected from the heads and sectioned with a Microslicer (D.S.K., DTK-1500E) in the coronal plane at 60 thickness. Sections were collected in serial order in chilled distilled water, soaked in a 1:1 solution of 100% glycerol and 8% paraformaldehyde (pH. 10.0) overnight at 4°C, then mounted on slides in the same solution and coverslipped (Senft, '90). Specimens were stored at 4°C in order to keep the dye labeling crisp for longer periods. The sections were observed with a Leitz epifluorescence microscope. Presence of growth cones at the end of most axons was taken as good evidence of complete staining.

Measurements and analysis

With the aid of a graticule scale in a 10 × eyepiece, the following two distances were estimated for each dye injection site under a 10 × objective: (1) distance from the center of the dye injection site to the front edge of the main bundle of growing callosal axons; and (2) distance of the front edge of the main bundle from the point at the midsagittal plane where callosal axons would or did cross (Glas, '75; Silver et al., '82; Wahlsten, '87). In addition, the distance between the center of the dye injection site and the midplane crossing point was also estimated for every injection site. When developing callosal axons never crossed midplane but formed an anomalous fiber bundle called the Probst bundle (Probst, '01), distance (1) was equal to the distance from the center of the injection site to the extremity of the medial border of this bundle at the level of the midplane crossing point, and distance (2) was the distance of the medial extremity of the bundle from midplane at the same level. Multiple regression methods (Marascuilo and Serlin, '88) were used to analyze the data in the same manner as for normal fetuses (Ozaki and Wahlsten, '92).

TABLE 2. Mean Values for Normal F_2 Hybrid[1] and Acallosal Mouse Strains

	Dye injection site			
	Frontal	Parietal	Temporal	Occipital
Body weight (g) at CC axon emergence				
Normal F_2	0.414	0.404	0.370	0.383
BALB/c and 129	0.387	0.390	0.350	0.390
CC axon growth rate (mm/g)				
Normal F_2	9.60	6.64	7.88	5.72
BALB/c and 129	9.69	5.58	7.43	5.74
Weight (g) when CC reached midplane				
Normal F_2	0.665	0.747	0.774	0.941
BALB/c and 129	0.642	0.762	0.766	0.948
Weight (g) when Probst bundle first appeared				
BALB/c and 129	0.66	0.72	0.80	0.97

[1]Data for B6D2F_2/J hybrid fetuses taken from Ozaki and Wahlsten ('92).

Reconstruction of growth cones and diagrams

With the aid of a graticule scale in a 10 × eyepiece, individual growth cones located entirely within a single section were drawn on paper under a 40 × dry objective. The base of the growth cone was defined as the point where the axon began to swell or bifurcated. The high density of labeled axons often made it difficult to identify the morphology of single growth cones within the main bundle. Consequently, all but a few of the growth cones chosen and analyzed in this study were located in front of or peripheral to the main bundle of labeled axons. The sizes of growth cones were quantitatively assessed by measuring their maximum length, maximum width, and two-dimensional surface area with an image analyzer (JAVA, Jandel Scientific).

Because callosal axons from each cortical region grow medially as well as longitudinally, it is very difficult to show the entire extent of an axon's trajectory by a few photo-graphs. Three-dimensional pathways of labeled callosal axons from individual dye injection sites were reconstructed into a two-dimensional diagram on paper with a graticule scale in a 10 × eyepiece and a 10 × objective.

RESULTS

For normal B6D2F_2/J hybrid fetuses measured under equivalent conditions (Ozaki and Wahlsten, '92), the chronological age (Age) expected for a given body weight (BW) is E(Age) = 14.65 + 2.42 BW. For the acallosal strains the difference between the actual age of a fetus and its age expected from this equation was

always 0.45 day or more and averaged more than 0.9 day for BALB /c and 129 fetuses, which means that the inbred fetuses lagged behind the normal comparison group by almost 1 full day of development (Wahlsten and Wainwright, '77). Because of these substantial strain differences, body weight rather than chronological age was used as the common measure for comparing genetically different fetuses (Wahlsten, '87).

As found previously (Ozaki and Wahlsten, '92), statistical results were not different for axons labeled with DiI or DiA. Although DiA diffuses faster than DiI (Chua et al., '90), the incubation times in all brains were adequate to allow both dyes to reach and fill the growth cones. In view of the varying numbers of dye injection sites in the fetuses (Table 1), the unit of analysis in this study is the injection site rather than the individual mouse. This introduces a small correlation among residuals for injection sites in the same animal but it also economizes greatly in the use of animals. Because of the unequal sample sizes and nonindependence of certain effects in the regression analyses as well as the large number of tests performed, only effects significant at $\alpha = 0.01$ are deemed worthy of attention.

Normal hybrids versus acallosal strains

Axon emergence from cortical cells. Because the first emergence of callosal axons from their cortical cells of origin was obscured by diffusion of the dye, regression equations provided an estimate of body size of a fetus when axons first emerge. Body weight (BW) can be predicted from the distance between the dye injection site and the leading edge of the main bundle of axons (ISMB) with the equation $E(BW) = B_0 + B_1ISMB$. When axons are just about to emerge, ISMB = 0 and the Y-intercept B_0 is the expected body weight when this event occurs. To compare acallosal strains with normal hybrids for each injection site, the body weight difference DIFF = BW — E(BW) was computed for a BALB/c or 129 fetus by inserting its ISMB value into the regression equation for the $B6D2F_2$ hybrids. For all 168 injection sites in fetuses weighing 0.7 g or less, when axons were about to emerge in acallosal strains, fetal body size was only 2.5 mg lower than for normal hybrids, which did not deviate significantly from zero ($t = —0.59$, $P > 0.5$). For all injection sites (Table 2), callosal axons of acallosal strains emerged at or slightly before the stage observed for normal hybrids; there certainly was no retardation of axon emergence.

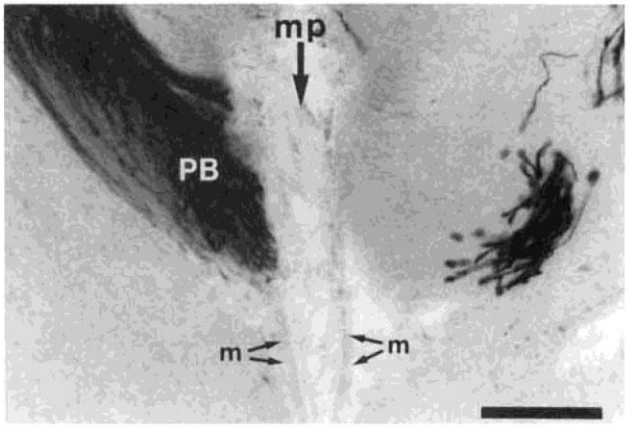

Fig. 1. Coronal section of an I/LnJ fetus at 0.71 g body weight with DiI in left frontal cortex and DiA in right parietal cortex. The callosal axons from frontal cortex have reached midplane (mp) and made contact with the meninges (m), forming the Probst bundle (PB), whereas those from parietal cortex are approaching midplane in this section and reach it in a more posterior section. Scale bar = 200 μm.

Axon growth rate. Axon growth was assessed in relation to body size for each injection site with equations of the type $E(ISMB) = B_0 + B_1BW$, where B_1 is the growth rate in millimeters of axon extension per gram body weight in-crease. For all 225 relevant data points the mean value of the difference from normal hybrids was 0.004 mm ($t = 0.27$, $P > 0.5$). The magnitude of the difference was nearly identical for BALB/c and 129 mice, and it was unrelated to body size. As is apparent in Table 2, axon growth rates were very similar in acallosal strains and normal hybrids; no major deficits were evident for any injection site, although growth rates differed substantially between sites.

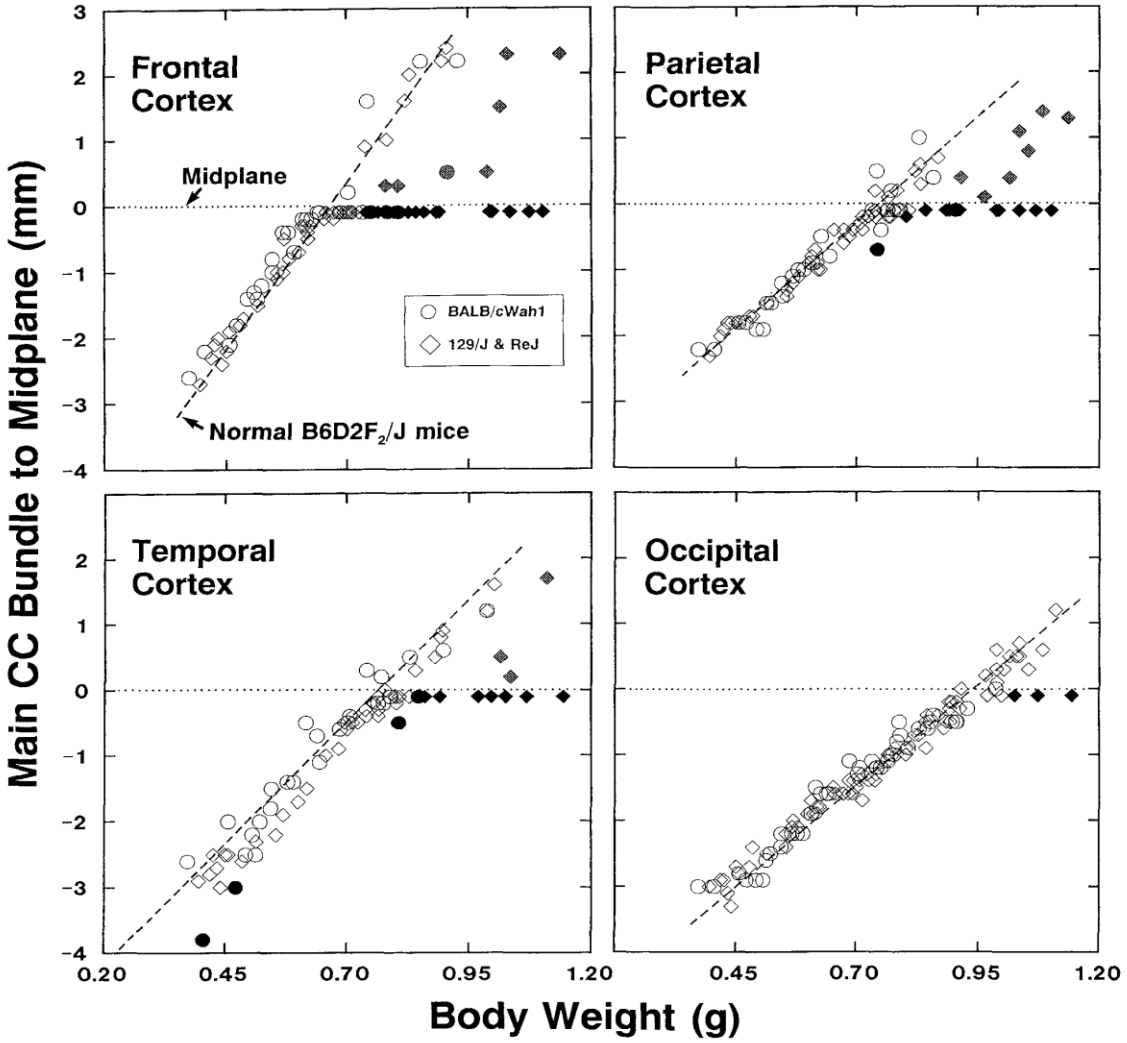

Fig. 2. Distance of the main bundle of callosal axons from midplane versus fetal body weight for the entire sample of 382 injection sites in BALB/c and 129 fetuses. The dashed lines are the regression equations for normal B6D2F$_2$ hybrids reported by Ozaki and Wahlsten ('92). The open symbols are animals within limits of normal development; the solid symbols indicate significantly delayed development in which observed distance is 80 μm (about two standard deviations) or more behind the value expected on the basis of fetal body weight; the shaded symbols are animals with delayed crossing of corpus callosum (CC) axons.

Arrival at midplane. Arrival of axons at the midsagittal plane was estimated from regression equations of the form E(BW) = B_0 + B_1CPMB, where CPMB is the distance in mm from the midplane crossing point to the leading edge of the main bundle of CC axons. Only instances in which the main bundle was not yet within 0.25 mm of midplane were used for this analysis. 'When the main bundle of CC axons reached midplane in acallosal strains, fetal body size was only 3 mg less than for hybrids at the same stage (t = —0.62, P > 0.5). As shown in Table 2, CC axons of these two acallosal strains arrived at midplane at about the same degree of fetal maturity as for normal hybrids.

TABLE 3. Frequency of Three Types of Callosal Development After the Arrival of Axons at Midplane in BALB/c and 129 Mice

	Dye injection site			
Type	Frontal	Parietal	Temporal	Occipital
Normal				
%	8.5	18.4	40.0	63.2
Cases	(4/47)	(7/38)	(14/35)	(12/19)
Partial agenesis				
%	29.8	28.9	25.7	21.1
Cases	(14/47)	(11/38)	(9/35)	(4/19)
Complete agenesis				
%	61.7	52.6	34.3	15.8
Cases	(29/47)	(20/38)	(12/35)	(3/19)

Thus, the statistical analysis based on linear regression methods indicates that BALB/c and 129 mice suffer no impairment of callosal axon emergence or growth toward the midplane crossing point. The litter of five I/LnJ mice included two small fetuses at 0.447 g and 0.467 g in which axons from all four cortical sites had already grown at least 0.5 mm, and there were three fetuses over 0.7 g in which frontal and parietal axons had already

reached midplane. Until they arrived at the deep cleft between the hemi-spheres (Fig. 1), axons of the I/LnJ mice also appeared to be normal.

Development after arrival at midplane. Development of callosal axons in BALB/c and 129 mice is portrayed graphically in Figure 2 by plotting individual values along with the lines of best fit for normal B6D2F_2/J hybrids from Ozaki and Wahlsten ('92). This figure clearly shows that the first deviation from the normal course of ontogeny occurred in these acallosal strains when CC axons reached the vicinity of the midsagittal plane on schedule (see also Fig. 1). The difficulties at midplane caused the diversity of the subsequent development of CC axons in BALB/c and 129 mice, and its essential pattern was the same among four different cortical sites (frontal, parietal, temporal, and occipital; Fig. 2). For ease of description, development of CC axons after arrival at midplane in these acallosal strains was categorized as three different types from the morphological point of view: (1) *normal:* developing callosal axons traversed the midplane without any signs of morphological disturbance and proceeded a distance in proportion with the body weight into the opposite hemisphere (Fig. 3D—F); (2) complete agenesis: callosal axons never crossed the midline and instead formed an anomalous fiber bundle (the aberrant callosal bundle of Probst, '01) adjacent to the longitudinal cerebral fissure (Fig. 3J—L); many axons in the bundle made contact with the meninges lining the bulge of the fissure but did not grow further along this tissue (Fig. 1); and (3) partial agenesis: developing callosal axons formed the Probst bundle but simultaneously a variable number of fibers traversed the midplane to enter into the opposite hemisphere (Fig. 3G—I). Crossing axons were of at least two kinds: the fibers that crossed the midline dorsally after forming the Probst bundle and then leaving it, and the fibers that directly traversed the midplane ventrally without participating in the formation of the bundle. The frequency (percentage of occurrence) of each type of development in BALB/c and 129 strains is given in Table 3 by cortical site. The percentage of complete agenesis type was highest for the frontal site (about 62%), decreased gradually for parietal and temporal sites, and was lowest for the occipital site (about 16%). On the other hand, the percent-age of normal type was lowest for the frontal site (about 9%) and highest for the occipital site (about 63%). The percent-age of partial agenesis type did not differ greatly among the four cortical sites and fell in the range between 21% (occipital site) and 30% (frontal site). These frequency differences are also apparent in Figure 2, in which the development of CC axons in BALB/c and 129 mice is divided into three groups according to the statistical analysis based on linear regression equations for normal hybrids. The frequency of each type of development after arrival at midplane shown in Table 3 is slightly different from that of the corresponding type in Figure 2 because of the difference of classification standards. The sum of percentages of complete and partial agenesis types for one cortical site in Table 3 is the probability that callosal axons from that site fail to find a path for crossing to the other side when they arrive at midplane. Therefore, the fact that this parameter drops from about 92% for the frontal site to about 37% for the occipital site clearly indicates that in BALB/c and 129 mice, there is recovery from or compensation for an early defect in the substrates of axon guidance at the midsagittal plane. This is confirmed by the occurrence of partial agenesis where there are both a Probst bundle and CC axons crossing midplane.

For 43 fetuses in BALB/c and 129 strains in which callosal axons from two different cortical regions had already arrived at the midline, we analyzed which combinations of three types of development were seen. There were ten fetuses in which more rostral axons showed a normal type of development. In all of these, development of more caudal axons was normal (Figs. 3D—F). This indicates that if axons from a more rostral region traverse the midplane normally, there is no interference with subsequent CC growth of those from more caudal regions. In 15 fetuses, on the other hand, in which more rostral axons exhibited a partial agenesis type, development of more caudal axons was the same type in 6 fetuses (Fig. 3H) but a normal type in 9 fetuses (Fig. 31). This observation implies that if axons from a more rostral region succeed in crossing to the other side before axons from a more caudal region reach the midsagittal region, more caudal axons can traverse the midplane normally. In the remaining 18 fetuses, more rostral axons showed a complete agenesis type of development. Development of axons from a more caudal region was the same type in 16 fetuses (Fig. 3K), but in 2 fetuses the types were partial agenesis and normal (Fig. 3L). This indicates that in BALB/c and 129 fetuses, callosal axons from a more caudal region can first cross midplane prior to those from a more rostral region, which is never seen in normal fetuses, in which callosal axons develop strictly according to a rostral-caudal gradient (Ozaki and Wahlsten, '92). This fact means further that callosal axons from any cortical region indeed

can traverse the midplane first, although the role is usually performed by the more rostral axons in normal development.

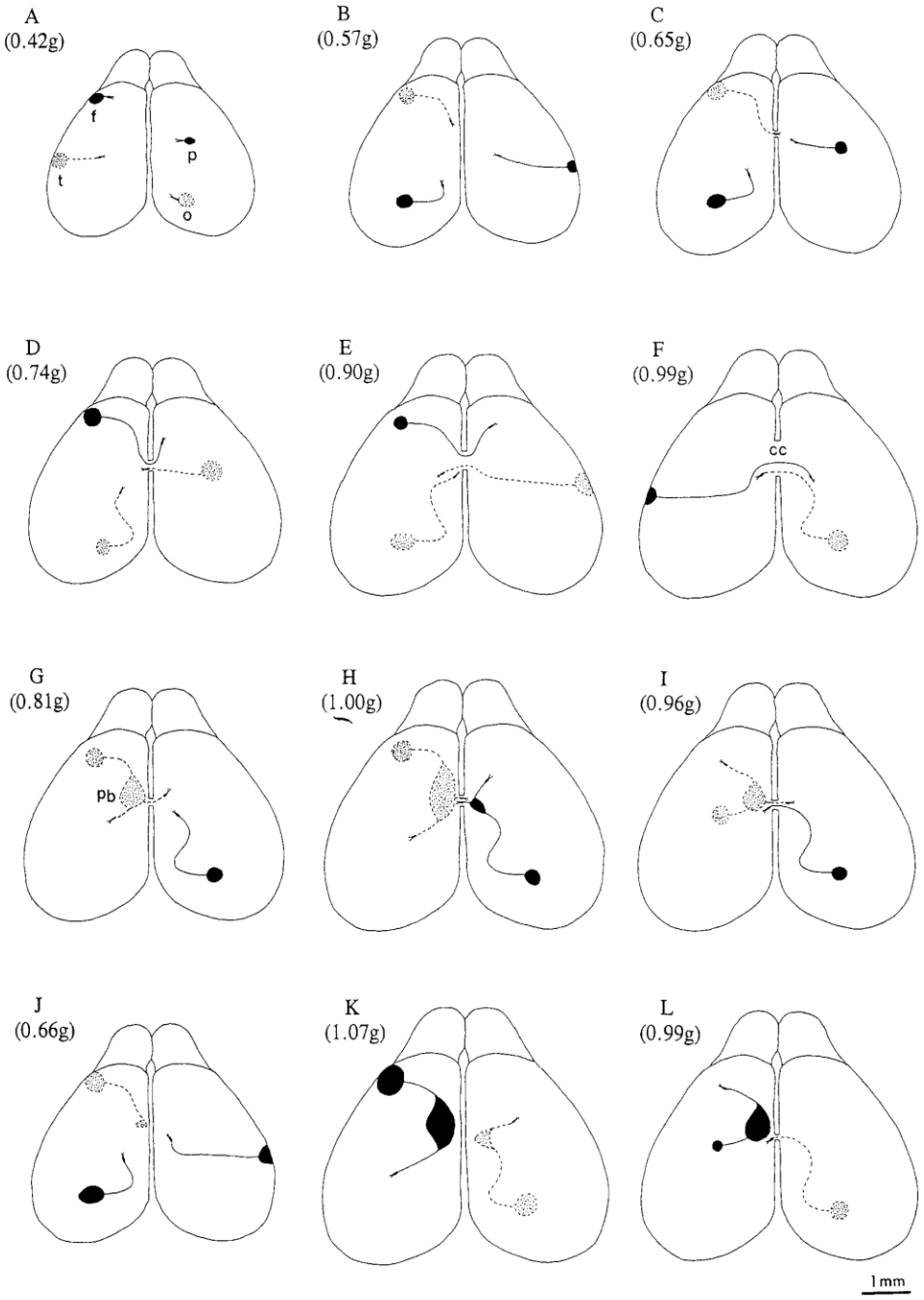

Fig. 3. Diagrams of strain 129 fetal brains illustrate development of callosal axons labeled with DiI (solid lines) or DiA (dotted lines). **A–C:** Early development until frontal axons arrive at midplane. **D–L:** Later development after their arrival at midplane. D–F, G–I, and J–L: Developmental features of CC axons from more caudal regions in cases in which more rostral axons exhibit normal, partial, and complete agenesis types of development, respectively. Letters f, p, t, and o stand for frontal, parietal, temporal, and occipital injection sites, respectively. cc, corpus callosum; pb, Probst bundle.

Probst bundle

Formation of the Probst bundle was first detected in fetuses of about 0.66 g body weight as an abnormal neuromatous accumulation of developing frontal axons (Fig. 3J) in the region where they would cross midplane in normal fetuses. At the initial stage of its formation, the bundle was rectangular in a cross section, and its fibers ran relatively parallel to one another (Fig. 1). As fetuses became older, however, the Probst bundle stretched longitudinally in a rostral as well as a caudal direction (see Fig. 3J and K), and it exhibited a rounder shape in a cross section, where axons whorled and took a tortuous and convoluted course. For more caudal injection sites, formation of the Probst bundle was observed first in fetuses of about 0.72 g (parietal), 0.80 g (temporal), or 0.97 g (occipital) body weight, which indicates that soon after reaching midplane and finding no bridge to the other side, CC axons from these sites begin to participate in the formation of the bundle according

to their rostral-caudal order (see Table 2). The first location of formation of the Probst bundle by these caudal axons was in the region where they would traverse the midline in normal fetuses.

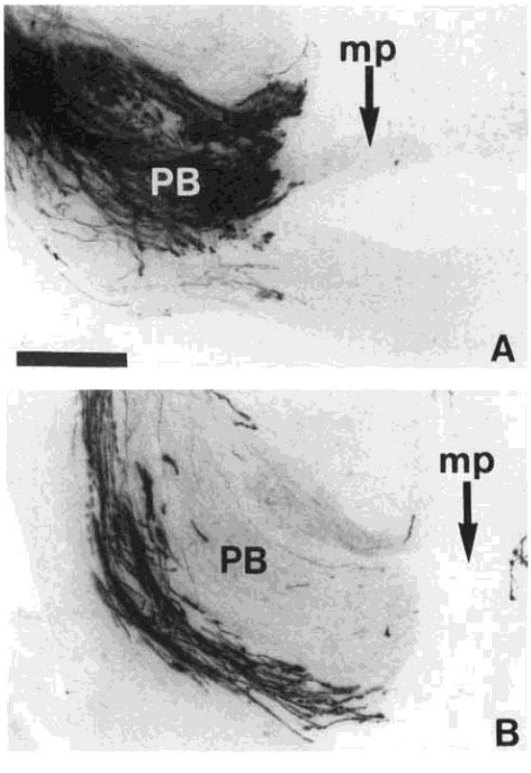

Fig. 4. Coronal sections of strain 129 fetal brains. **A:** DiA-labeled parietal callosal fibers forming the Probst bundle (PB) in a fetus of 1.07 g body weight. **B:** DiA-labeled temporal callosal fibers forming the Probst bundle (PB) in a fetus of 1.03 g body weight. Note a difference in the dorsal-ventral location of labeled axons within the bundle. Arrows indicate midplane (mp). Scale bar = 200 μm.

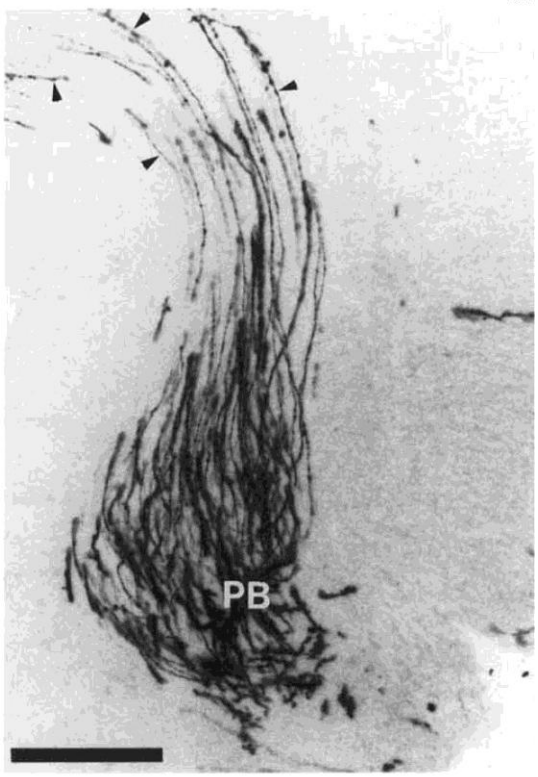

Fig. 5. Coronal section of a strain 129 fetus at 0.89 g body weight, showing DiA-labeled fibers (arrowheads) from frontal cortex that leave the Probst bundle (PB) ipsilaterally to proceed in the cortical white matter of the left parietal cortex. Midplane is to the right. Scale bar = 200 μm.

The topographic features of axons within the bundle differed greatly, depending on their origins; whereas axons from medial cortical regions (frontal, parietal, or occipital) occupied mainly its dorsomedial sector (Fig. 4A), those from the lateral region (temporal) were located exclusively in its ventrolateral sector (Fig. 4B). A similar topographic arrangement of axons in the Probst bundle has been found in congenitally (Ozaki and Shimada, '88) or surgically induced (Lent, '84) acallosal adult brain. These observations imply that in spite of their entirely aberrant location and trajectories, callosal axons in the bundle still retain some topographic information.

There were at least three kinds of fibers that left the Probst bundle:

1. Commissural fibers left the bundle medially to cross midplane and enter into the opposite hemisphere (Fig. 3G—I). The level at which these fibers traversed the mid-plane was often caudal to that where axons from the same cortical region would cross in normal fetuses. Axons from the Probst bundle usually used the hippocampal commissure as a bridge for crossing.

2. Some fibers left the bundle dorsolaterally to proceed in the ipsilateral cortical white matter (Fig. 5; see also Fig. 3G—I,K,L). These fibers were first seen in about 0.7 g, 0.8 g, 0.85 g, and 1.0 g fetuses for frontal, parietal, temporal, and occipital injections, respectively, which indicates that they stayed in the Probst bundle for only a few hours. Once the axons in this bundle had diverted into an ipsilateral growth pattern, they were never seen to turn back toward the midplane. In adult acallosal brains, these fibers are functional (Lefkowitz et al., '91) and are known to form ipsilateral corticocortical connections eventually in a similar topographical arrangement as the intrinsic association fibers (Ozaki et al., '89).

3. Other fibers left the bundle ventrally to descend ipsilaterally in septal tissue along the lateral wall of the longitudinal fissure (not shown). Although these fibers did not cross midplane in situ, their final destinations were not clear.

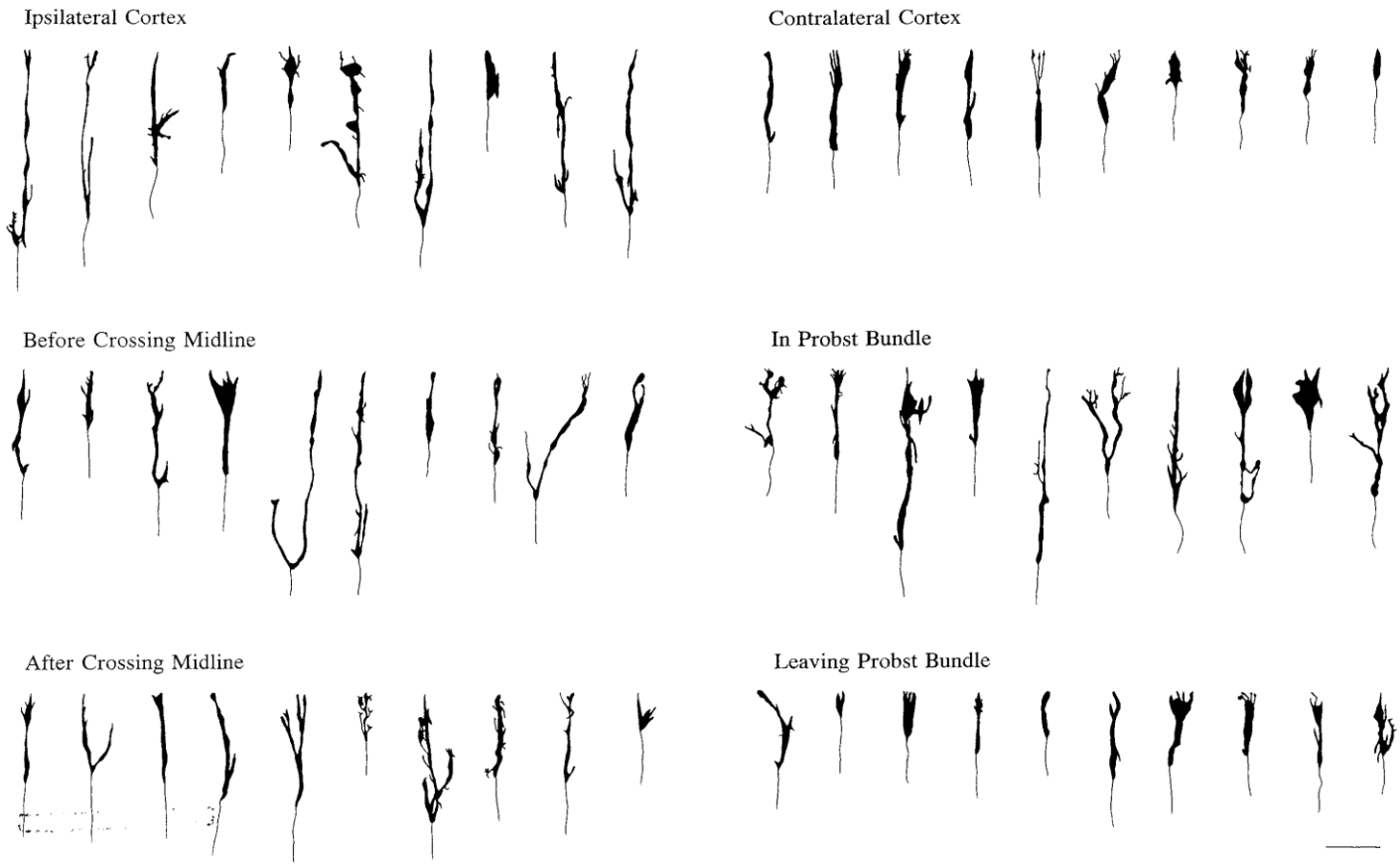

Fig. 6. Examples of growth cones in each cortical location. The growth cones displayed for each group were selected from a much larger sample using a random number table. Scale bar = 20 μm.

Growth cone morphology

Growth cones of developing callosal axons in BALB/c and 129 fetuses were very diverse in size and morphology (Fig. 6); some were club-shaped, with a small, round swelling at the tip of the axon, whereas others were elaborate and branched with prominent filopodia. The length of growth cones varied from about 10μm to 100 μm. Regardless of the size or morphology, almost all growth cones extended filopodia. Filopodia arising from the body or front edge of the growth cone generally pointed in the direction of the axon, whereas those arising from its proximal end or from the axon itself sometimes were directed backward. Filopodia number varied from only a few to as many as ten, giving a hairy appearance to the axon tip. Filopodia ranged in length from small nubs of about 1 μm to branches up to 70 μm.

TABLE 4. Measures of Growth Cones in Each of Six Zones of Cerebral Cortex of BALB/c and 129 Mice[1]

Zone	Sample size	Area (μm²)	Length (μm)	Width (μm)
Ipsilateral cortex	96	103.2[a]	47.1[a]	9.7[a]
Before crossing midplane	96	97.3[a]	40.8[a]	9.3[a,b]
After crossing midplane	96	76.1[b]	32.8[b]	7.4[b]
Contralateral cortex	96	74.6[b]	29.7[b]	7.4[a,b]
In Probst bundle	96	108.9[a]	45.0[a]	12.6
Leaving Probst bundle	96	69.1[b]	28.9[b]	8.0[a,b]

[1]For a particular measure, groups with the same superscript letter are not significantly different according to a Newman-Keuls post hoc test with α = 0.01. This test requires equal sample sizes for all groups, so observations were randomly discarded to equate sample sizes at the smallest group size (n = 96). Probability values are only approximate because more than one growth cone in a region was often drawn for one animal.

In a previous study on normal hybrid fetuses (Ozaki and Wahlsten, '92), we observed a wide range of growth cone size and morpholou as well as differences along the callosal pathway; larger growth cones with more complex morphologies were more often seen in callosal axons proceeding within the ipsilateral white matter as well as in the vicinity of the midplane region, whereas growth cones of callosal axons in the white matter of the contralateral hemisphere were somewhat smaller and simpler. For the BALB/c and 129 fetuses in this study, 974 growth cones drawn on paper were classified according to their locations along the callosal pathway. The diagram of a random sample from each location (Fig. 6) reveals a wide variety of morphologies, whereas the mean measurements (Table 4) denote small but statistically significant differences between locations.

Altogether, these observations indicate that callosal growth cones of acallosal mice have normal sizes and shapes.

DISCUSSION

The present results demonstrate that the emergence of CC axons from cortical cells and their subsequent growth toward the midplane crossing point are evidently normal in acallosal strain mice and that the first deviation of their development from the normal course of ontogeny occurs when the axons reach the midplane region on schedule. These findings substantiate the current hypothesis that the processes of axon guidance are normal in these mice until the fibers approach the midsagittal plane and that the anatomical problem with callosal agenesis does not reside in the cells of origin or the axons themselves (Silver et al., '82; Wahlsten, '87). Our present morphometric results on growth cones in BALB/c and 129 strains also support this idea.

The analysis of axon development after arrival at mid-plane in BALB/c and 129 fetuses has revealed that the frequency of formation of the Probst bundle by CC axons dramatically decreases in the rostral-caudal sequence of cortical regions of their origin; it was highest for frontal axons that arrived at the midsagittal plane first and lowest for occipital axons that were the last to reach it. This finding confirms a previous claim (Wahlsten, '87) that in these fetuses the midline substrate defect responsible for CC agenesis occurs relatively early in development and that there is recovery from or compensation for this early substrate defect in later development. Once recovery or compensation occurs, callosal axons that arrive at midplane later cross normally without participating in the formation of the Probst bundle, and some axons in the Probst bundle also manage to traverse the midline. The rostral-caudal sequence of callosal development (Ozaki and Wahlsten, '92) and the recovery from the defect of midline structures in later development explains well why in adult mice and human brains with partial CC agenesis the Probst bundle is always seen more rostrally than caudally (King, '36; Loeser and Alvord, '68). Thus, partial absence or reduced size of the adult CC is not the result of arrested development of callosal axons at the midline, as was previously believed (Loeser and Alvord, '68; Wahlsten, '81; Ozaki et al., '87), but is the result of compensationary processes in development of midline structures and plastic processes of CC axons (Wahlsten, '87).

In acallosal strain fetuses recovery from or compensation for an early defect in the substrates for CC axon growth at midplane occurs when the HC becomes large enough that callosal axons can find a path to traverse a gap between the hemispheres by growing across the dorsal surface of this commissure (Wahlsten, '87). The CC at midplane can then grow rapidly when incoming axons reach this bridgehead. Apparently, the earlier the HC achieves a sufficient size, the larger the size of the CC will be in the adult. It is therefore interesting that during the recovery process, callosal axons in BALB /c and 129 fetuses do not always obey the strict rostral-caudal sequence that CC axons in normal fetuses display when they cross midplane (Ozaki and Wahlsten, '92); axons from a more caudal region sometimes grow across the midline prior to those from a more rostral region. This probably occurs because, regardless of cortical regions of their origin, any callosal axons can traverse a delayed bridgehead at the HC first if they arrive at it first. Tracing CC axons in adult mice with partial CC agenesis confirms that even a very small CC contains axons from many regions of the cortex (Ozaki et al., '87; Olavarria et al., '88).

In BALB/c mice the frequency of El9 fetuses with no CC at midplane is about the same as in adults (Wahlsten, '87). This indicates that there is a critical period for CC formation via the HC bridge. When reaching midplane and failing to locate a bridge across the gap between the hemispheres, CC axons inevitably form the Probst bundle, but they stay within this bundle only for a few hours. Once the axons leave the bundle to proceed into the ipsilateral white matter, they never turn back toward the midplane. Therefore, unless the HC has achieved a sufficient size before the time that CC axons from all cortical regions emerge from the Probst bundle into an ipsilateral growth pattern, no CC will ever form. In fact, there is a close association between percentage of adults with total CC agenesis and degree of retarded formation of the HC among several mouse strains that suffer absence of the CC in the adult (Wahlsten and Bulman-Fleming, '90); I /Ln strain mice never have a CC in the adult because they suffer severe impairment of the HC formation, so severe that this commissure usually does not reach its normal adult size (Livy and Wahlsten, '91).

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